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(54) Title: MACROPHAGE ACTIVATING FACTORS DERIVED FROM CLONED VITAMIN D BINDING PROTEIN (57) Abstract Vitamin D-binding protein (Gc protein) and its small domain (approximately 1/5 of the Gc peptide also known as domain III) were cloned via a baculovirus vector. The cloned Gc protein and the cloned domain (Cd) peptide were treated with immobilized β -galactosidase and sialidase to yield macrophage activating factors, GcMAFc and CdMAF, respectively. These cloned macrophage activating factors and GcMAF may be used for therapy of cancer, HIV-infection and osteoporosis, and may also be used as an adjuvant for immunization and vaccination. Methods and an antibody-sandwich ELISA kit were developed to detect serum or plasma α -N-acetylgalactosaminidase in cancer and HIV-infected patients.		

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Macrophage Activating Factors Derived From Cloned Vitamin D Binding Protein

SPECIFICATION

FIELD OF THE INVENTION

This invention relates to potent macrophage activating factors, prepared by oligosaccharide digestion of the cloned vitamin D binding protein (Gc protein) and the cloned Gc protein domain III, and the use of these macrophage activating factors for various cancers, HIV-infection and osteoporosis, and as a adjuvant for immunization and vaccination and also development of diagnostic and prognostic methods utilizing the specific enzyme, α -N-acetylgalactosaminidase detected in the blood stream of cancer and HIV-infected patients.

TABLE OF TERMS

Gc protein	Vitamin D ₃ -binding protein
MAF	macrophage activating factor
GcMAF	Gc protein-derived macrophage activating protein
GcMAFc	cloned Gc protein-derived macrophage activating factor
Gc domain III	domain III region of Gc protein
CdMAF	cloned domain III-derived macrophage activating factor
NagAg	α -N-acetylgalactosaminidase as an antigen

SUMMARY OF THE INVENTION

Vitamin D-binding protein (Gc protein) and its small domain (approximately 18% length of the Gc peptide also known as domain III) were cloned via a baculovirus vector. The cloned Gc protein and the cloned domain (Cd) peptide were treated with immobilized β -galactosidase and sialidase to yield macrophage activating factors, GcMAFc and CdMAF, respectively. These cloned macrophage activating factors and GcMAF are to be used for cancer therapy, HIV-infection and osteoporosis, and may also be used as a adjuvant for immunization and vaccination. Methods and an antibody-sandwich ELISA kit were developed to detect serum or plasma α -N-acetylgalactosaminidase in cancer and HIV-infected patients and used as diagnostic and prognostic indices.

DESCRIPTION OF THE DRAWINGS

Other objects and many attendant features of this invention will become readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Fig. 1a is a schematic illustration of the formation of macrophage activating factor (MAF).

Fig. 1b is a schematic illustration of the deglycosylation of Gc protein in a cancer or HIV-infected patient's blood stream.

Fig. 2 shows the amino acid sequence of cloned GcMAF which is SEQ ID NO:1 which is the entire cloned Gc protein.

Fig. 3 shows the construction of the DNA fragment encoding the leader sequence of EcoRI fragment E1 and domain III regions of the Gc protein; A, the entire cDNA for Gc protein; B, the construct to be inserted into the non-fusion vector; the shaded area indicates the compressed regions of about 1,000 base pairs (bp).

Fig. 4 shows the 89 amino acid sequence, SEQ ID NO:2, of the cloned domain III (CdMAF₁), using the non-fusion vector.

Fig. 5 shows the baculovirus fusion vector for cloning the domain III of Gc protein.

Fig. 6 shows the 94 amino acid sequence, SEQ ID NO:3, of the cloned domain III (CdMAF₂), using the fusion vector.

Fig. 7 shows the correlation between plasma α -N-acetylgalactosaminidase activity and tumor burden (total cell counts) in the peritoneal cavity of Ehrlich ascites tumor.

Fig. 8A shows the therapeutic effect of GcMAF in accordance with the present invention on adult persons suffering from prostate cancer.

Fig. 8B shows the therapeutic effect of GcMAF in accordance with the present invention on adult persons suffering from breast cancer.

Fig. 8C shows the therapeutic effect of GcMAF in accordance with the present invention on adult persons suffering from colon cancer.

BACKGROUND OF THE INVENTION

A. Inflammatory Response Results in Activation of Macrophages

Inflammation results in the activation of macrophages. Inflamed lesions release lysophospholipids. The administration into mice of small doses (5-20 μ g/mouse) of lysophosphatidylcholine (lyso-Pc) and other lysophospholipids induced a greatly enhanced phagocytic and superoxide generating capacity of macrophages (Ngwenya and Yamamoto, Proc. Soc. Exp. Biol. Med. 193:118, 1990; Yamamoto et al., Inf. Imm. 61:5388, 1993; Yamamoto et al., Inflammation. 18:311, 1994).

This macrophage activation requires participation of B and T lymphocytes and serum vitamin D binding protein (DBP; human DBP is known as Gc protein). In vitro activation of mouse peritoneal macrophages by lyso-Pc requires the step-wise modification of Gc protein by β -galactosidase of lyso-Pc-treated B cells and sialidase of T cells to generate the macrophage activating factor (MAF), a protein with N-acetylgalactosamine as the remaining sugar moiety (Fig 1a) (Yamamoto et al., Proc. Natl. Acad. Sci. USA. 88:8539, 1991; Yamamoto et al., J. Immunol. 151:2794, 1993; Naraparaju and Yamamoto, Immunol. Lett. 43:143, 1994). Thus, Gc protein is a precursor for MAF.

Incubation of Gc protein with immobilized β -galactosidase and sialidase generates a remarkably high titered MAF (GcMAF) (Yamamoto et al., Proc. Natl. Acad. Sci. USA. 88:8539, 1991; Yamamoto et al., J. Immunol. 151:2794, 1993; Naraparaju and Yamamoto, Immunol. Lett. 43:143, 1994; US Patent # 5,177,002; US Patent # 5,326,749). Administration of a minute amount (10 pg/mouse; 100 ng/human) of GcMAF resulted in greatly enhanced phagocytic and superoxide generating capacities of macrophages.

When peripheral blood monocytes/macrophages (designated as macrophages hereafter) of 326 patients bearing various types of cancer were treated in vitro with 100 pg GcMAF/ml, the macrophages of all cancer patients were activated for phagocytic and superoxide generating capacity. This observation indicates that cancer patient macrophages are capable of being activated. However, the MAF precursor activity of plasma Gc protein was lost or reduced in approximately 70% of this cancer patient population. Lost or reduced precursor activity of Gc protein was found to be due to deglycosylation of plasma Gc protein by α -N-acetylgalactosaminidase detected in the

cancer patient's blood stream. Deglycosylated Gc protein cannot be converted to MAF (Fig. 1b). Thus, the loss of the MAF precursor activity of Gc protein prevents generation of MAF. Therefore, macrophage activation cannot develop in certain cancer patients. Since macrophage activation is the first step in the immune development cascade, such cancer patients become immunosuppressed. This may explain at least in part why cancer patients die from overwhelming infection.

Similarly, when peripheral blood macrophages of 196 HIV-infected/AIDS patients were treated in vitro with 100 pg GcMAF/ml, the macrophages of all patients were activated for phagocytic and superoxide generating capacity. However, the MAF precursor activity of plasma Gc protein was low in approximately 35% of the HIV-infected patient population. As in cancer patients, these patients' plasma Gc protein is deglycosylated by α -N-acetylgalactosaminidase detected in HIV-infected patients. This mechanism explains why advanced HIV-infected (AIDS) patients are severely immunosuppressed.

Both cancer and HIV-infected patients having severely decreased precursor activity of plasma Gc protein carried large amounts of α -N-acetylgalactosaminidase while patients having moderately decreased precursor activity had moderate levels of plasma α -N-acetylgalactosaminidase activities. Patients with high precursor activity, including asymptomatic HIV-infected patients, had low but significant levels of plasma α -N-acetylgalactosaminidase activity. Since a large amount (260 μ g/ml) of Gc protein exists in the blood stream, a low level of the enzyme does not affect the precursor activity. Nevertheless, α -N-acetylgalactosaminidase activity was found in plasmas of all cancer and HIV-infected patients and had an inverse correlation with the precursor activity of their plasma Gc protein. Thus, increase in patient serum or plasma α -N-acetylgalactosaminidase activity is responsible for decrease in the precursor activity of plasma Gc protein. These observations led me to propose that serum or plasma α -N-acetylgalactosaminidase plays a role in immunosuppression in cancer and HIV-infected/AIDS patients. Thus, patient serum or plasma α -N-acetylgalactosaminidase activity can serve as a diagnostic and prognostic index.

B. A Defect in Macrophage Activation Cascade Manifests Osteoporosis

The inflammation-primed macrophage activation process appears to be the major macrophage activation cascade, which is shared by other phagocytes such as osteoclast

and monocytes. A defect in the inducible β -galactosidase of B lymphocytes in the macrophage activation cascade causes dysfunctional osteoclasts leading to manifestation of osteoporosis (Yamamoto *et al.*, *J. Immunol.* 152:5100, 1994), lacking or delaying bone marrow formation in newborn.

Autosomal recessive osteoporosis is characterized by an excess accumulation of bone throughout the skeleton as a result of dysfunctional osteoclasts, resulting in reduced bone resorption (Marks, *Clin. Orthop.* 189:239, 1984). In animal models of osteoporosis, depending on the degree of osteoclast dysfunction, marrow cavity development and tooth eruption are either delayed or more commonly absent (Marks, *Am. J. Med. Genet.* 34:43, 1989). In human infantile osteoporosis, death occurs within the first decade of life usually overwhelming infection (Reeves, *Pediatrics.* 64:202, 1979), indicating immunosuppression. Accumulated evidence suggests that deficient or dysfunctional osteoclasts in osteopetrotic mammals including humans are often accompanied by deficiencies or dysfunctions of macrophages. The studies of the present inventor on the activation of both osteoclasts and macrophages in the osteopetrotic mutations revealed that osteoclasts and macrophages can be activated by a common signaling factor, the macrophage activating factor and that a defect in β -galactosidase of B cells incapacitates the generation process of macrophage activating factor (Yamamoto *et al.*, *J. Immunol.* 152:5100, 1994). Since GcMAF and its cloned derivatives bypass the functions of lymphocytes and Gc protein and act directly on macrophages and osteoclasts, administration of these factors into osteopetrotic hosts should rectify the bone disorder. In fact the present inventor has recently found that four administrations of GcMAF or cloned macrophage activating factor (GcMAFc) (100 pg/week) to the *op* mutant mice beginning at birth for four weeks resulted in the activation of both macrophages and osteoclasts and subsequent resorption of the excess skeletal matrix and increased size of marrow cavity.

C. Therapeutic Application of GcMAF and its Cloned Derivatives on Cancer

Despite defects in the macrophage activation cascade in cancer, HIV-infected and osteopetrotic patients, GcMAF bypasses the functions of lymphocytes and Gc protein and acts directly on macrophages (or osteoclasts) for activation. Macrophages have a potential to eliminate cancerous cells and HIV-infected cells when activated. When cancer patients were treated with 100 ng GcMAF/patient weekly for several months,

GcMAF showed remarkable curative effects on a variety of human cancer indiscriminately.

Instead of obtaining of GcMAF from human blood source, it can be obtained from the cloned Gc protein or its small domain responsible for macrophage activation. The cloned Gc protein requires an eukaryotic vector/host capable of the glycosylation of the cloned products. The Gc protein with a molecular weight of approximately 52,000 and 458 amino acid residues) is a multi-functional protein and carries three distinct domains (Cooke and Haddad, Endocrine Rev., 10:294, 1989).

Domain I interacts with vitamin D while domain III interacts with actin (Haddad et al., Biochem., 31:7174, 1992). Chemically and proteolytically fragmented Gc protein enabled me to indicate that the smallest domain, domain III, contains 80 amino acid residues including the site for glycosylation, an essential peptide for macrophage activation. Accordingly, I cloned both Gc protein and the entire domain III peptide, by the use of a baculovirus vector and an insect host, and treated them with the immobilized β -galactosidase and sialidase to yield potent macrophage activating factors, designated GcMAFc and CdMAF, respectively. Like GcMAF, these cloned GcMAFc and CdMAF appear to have remarkably curative effects on a variety of cancers.

**D. A potent adjuvant activity of GcMAF for
immunization with antigens or vaccines**

Macrophages are antigen presenting cells. Macrophages activated by GcMAF rapidly phagocytize target antigens or cells and presented the processed antigens to antibody producing cells. I observed a rapid development of a large amount of antibody secreting cells immediately (1 to 4 days) after inoculation of small amount of GcMAF (100 pg/mouse) and sheep erythrocytes (SRBC). This finding indicates that GcMAF and its cloned derivatives, GcMAFc and CdMAF, should serve as a potent adjuvant for immunization and vaccination.

**DESCRIPTION OF THE METHODS FOR GENE CLONING
FOR MACROPHAGE ACTIVATING FACTORS**

A. Cloning of the cDNA of Gc Protein into an Insect Virus.

A full length cDNA encoding the human Gc protein was isolated from a human liver cDNA library in bacteriophage λ gt11 (Clontech, Palo Alto, CA) by the use of a pico Blue_{TM} immunoscreening kit available from Stratagene of La Jolla, CA. The

baculoviral expression system in the insect cells takes advantages of several facts about the polyhedron protein: (a) it is expressed to very high levels in infected cells where it constitutes more than half of the total cellular protein late in the infection cycle; (b) it is nonessential for infection or replication of the virus, meaning that the recombinant virus does not require any helper function; (c) viruses lacking the polyhedron gene have distinct plaque morphology from viruses containing the cloned gene; and d) unlike bacterial cells, the insect cell efficiently glycosylates the cloned gene products.

One of the beauties of this expression system is a visual screen allowing recombinant viruses to be distinguished and quantified. The polyhedron protein is produced at very high levels in the nuclei of infected cells late in the viral infection cycle. Accumulated polyhedron protein forms occlusion bodies that also contain embedded virus particles. These occlusion bodies, up to 15 μm in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under a light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish the recombinant virus from a wild-type virus, the transfection supernatant (recombinant containing virus lysate) is plaqued onto a monolayer of insect cells. Plaques are then screened under a light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies.

In addition, the baculoviral expression system uses a helper-independent virus that can be propagated to high titers in insect cells adapted for growth in suspension cultures, making it possible to obtain large amounts of recombinant protein with relative ease. The majority of the overproduced protein remains soluble in insect cells by contrast with the insoluble proteins often obtained from bacteria. Furthermore, the viral genome is large (130 kbp) and thus can accommodate large segments of foreign DNA.

1) Choice of baculoviral vector.

All available baculoviral vectors are pUC-based and confer ampicillin resistance. Each contains the polyhedron gene promoter, variable lengths of polyhedron coding sequence, and insertion site(s) for cloning the foreign gene of interest flanked by viral sequences that lie 5' to the promoter and 3' to the foreign gene insert. These flanking sequences facilitate homologous recombination between the vector and wild-type baculoviral DNA (Ausubel *et al.*, Current Protocols in Mol. Biol. 1990). The major

consideration when choosing the appropriate baculoviral expression vector is whether to express the recombinant as a fusion or non-fusion protein. Since glycosylation of the Gc peptide requires a leader signal sequence for transfer of the peptide into the endoplasmic reticulum, the cDNA containing initiation codon (-16 Met) through the leader sequence to the +1 amino acid (Ieu) of the native Gc protein should be introduced to the non-fusion vector with a polylinker carrying the EcoRI site, pLV1393 (Invitrogen, San Diego, CA).

During partial digestion of the cDNA for Gc protein in λ gt11 with EcoRI enzyme, a full length Gc cDNA with EcoRI termini was isolated electrophoretically, mixed with EcoRI-cut pVL1393, and ligated with T4 ligase. This construct in the correct orientation should express the entire Gc peptide, a total of 458 amino acids (Fig. 2). To obtain the correct construction, competent *E. coli* HB101 cells were transformed with pVL vector and selected for transformants on Luria broth agar plates containing ampicillin (LB/ampicillin plates). The DNA was prepared for the sequencing procedure to determine which colony contains the insert or gene with the proper reading orientation, by first searching for the 3' poly A stretch. The clones with 3' poly A (from the poly A tail of mRNA) were then sequenced from the 5' end to confirm the correct orientation of the full length DNA for the Gc peptide.

2) Co-transfection of insect cells with the cloned plasmid DNA and wild-type viral DNA

A monolayer (2.5×10^6 cells in each of 25-cm² flasks) of *Spodoptera frugiperda* (Sf9) cells was co-transfected with a cloned plasmid (vector) DNA (2 μ g) and a wild-type (Ac-MNPV) baculoviral DNA (10 μ g) in 950 μ l transfection buffer (Ausubel *et al.*, *In Curr Protocols in Mol. Biol.* 1990). When the cells were cultured for 4 or 5 days, the transfection supernatant contained recombinant viruses.

3) Identification of recombinant baculovirus

The co-transfection lysates were diluted 10^4 , 10^5 or 10^6 and plated on Sf9 cells for cultivation for 4 to 6 days. After the plaques were well formed, plaques containing occlusion-negative cells were identified at a frequency of 1.3%. Several putative recombinant viral plaques were isolated and twice re-plaque for purification. Pure recombinant viral plaque clones were isolated.

B. Analysis of Protein of Interest from Recombinant Baculovirus**1) Preparation of recombinant virus lysate**

An insect cell Sf9 monolayer (2.5×10^6 cells per 25-cm^2 flask) was infected with a recombinant virus clone and cultured in 5 ml GIBCO serum-free medium (from GIBCO Biochemicals, Rockville, MD) or medium supplemented with 0.1% egg albumin to avoid contamination of serum bovine vitamin D binding protein. The culture flasks were incubated at 27°C and monitored daily for signs of infection. After 4 to 5 days, the cells were harvested by gently dislodging them from the flask and the cells and culture medium were transferred to centrifuge tubes and centrifuged for 10 min at $1000 \times g$, 4°C . To maximize infection for recombinant protein production, Sf9 cells were grown in a 100-ml spinner suspension culture flask with 50 ml complete medium up to about 2×10^6 cells/ml. The cells were harvested, centrifuged at $1000 \times g$ for 10 min and re-suspended in 10 to 20 ml serum-free medium containing recombinant virus at a multiplicity of infection (MOI) of 10. After 1 hour of incubation at room temperature, the infected cells were transferred to a 200-ml spinner flask containing 100 ml serum-free medium and incubated for 40 hr. More than 40% of the protein secreted was the protein of interest. The protein in the supernatant was isolated.

2) Qualitative estimation of the protein of interest

Coomassie Blue staining of the SDS-polyacrylamide gel, loading 20 to $40 \mu\text{g}$ total cell protein per lane, was used to estimate the quantity of expressed protein. Because the samples contain cellular proteins, the recombinant protein was readily detected by comparison with uninfected cellular proteins.

3) Enzymatic conversion of the cloned Gc protein to macrophage activating factor (GcMAFc).

The cloned Gc protein ($2 \mu\text{g}$) with a molecular weight of 52,000 and 458 amino acid residues (Fig. 2) was isolated by a vitamin D-affinity column (Link et al. *Anal. Biochem.* 157:262, 1986) and treated with immobilized β -galactosidase and sialidase. The resultant cloned macrophage activating factor (GcMAFc) was added to mouse and human macrophages and assayed for phagocytic and superoxide generating capacity. Incubation of macrophages with 10 pg GcMAFc/ml for 3 hours resulted in a 5-fold increased phagocytic activity and a 15-fold increase in the superoxide generating capacity of macrophages.

C. Subcloning of a Domain Required for Macrophage Activation**I. Cloning procedure I: Non-fusion vector.****1) Cloning the domain responsible for macrophage activation (CdMAF)**

The entire cDNA sequence for Gc protein in λ gt11, including 76 bp of the upstream 5' flanking region and 204 bp of the 3' flanking stretch, was fragmented by EcoRI to yield four restriction fragments designated E1, 120; E2, 314; E3, 482; and E4, 748 bp, respectively. Each was cloned into the EcoRI site of the plasmid pSP65 from Promega (Madison, WI) by the method of Cooke and David (*J. Clin. Invest.*, 76 2420, 1985). Although I found that a region less than one half of the domain III was found to be responsible for macrophage activation, small segments less than 40 amino acid residues cannot be expressed in the insect cells. Moreover, short peptides are rapidly degraded by proteases in human plasma, and thus are not clinically useful. Accordingly, the entire domain III (approximately 80 amino acid residues) should be subcloned into an insect virus where I anticipate the efficient production and glycosylation of the peptide in the infected cells.

2) Subcloning cDNA fragment into the polyhedron gene of baculovirus

Since the glycosylation of a peptide requires a leader signal sequence for transfer of the peptide into the endoplasmic reticulum, the DNA segment of E1 containing the initiation codon (-16 Met) through the leader sequence to the +1 amino acid (Leu) of the native Gc protein should be introduced into the vector. Because this segment carries the initiation codon for the Gc protein, non-fusion vector, pVL1393 (Invitrogen, San Diego, CA) was used. A segment containing the initiation codon-leader sequence of the cDNA clone E1 and a cDNA segment coding for 85 C-terminal amino acids (the entire domain III) plus 3' non-coding stretch of the cDNA clone E4 were ligated together and cloned into the EcoRI site of the insect virus pVL vector. To achieve this construct, both E1 and E4 DNA were fragmented with HaeIII to yield two fragments each; E1hl (87 bp), E1hs (33 bp) and E4hs (298 bp), E4hl (450 bp), respectively. Both the larger fragments E1hl and E4hl were isolated electrophoretically, mixed with EcoRI-cut pVL, and ligated with T4 ligase, as shown in Fig. 3. This construct in the correct orientation should express the entire domain III, a total of 89 amino acids, including the

4 amino acids of E1hl, also referred to herein as CdMAF₁ as shown in Fig. 4. To obtain the correct construction, competent *E. coli* HB101 cells are transformed with the pVL vector and selected for transformants on LB/ampicillin plates. DNA was prepared for sequencing procedures to determine which colony contains the construct with proper reading orientation by first searching for the 3' poly dA stretch. Those clones with 3' poly dA (from the poly A tail of mRNA) were then sequenced from the 5' end to confirm the correct orientation of the E1hl fragment. I found that the vector contains the entire construct (domain III) in the correct orientation.

3) Isolation of recombinant baculovirus, purification of the cloned domain peptide (Cd) and enzymatic generation of the cloned macrophage activating factor (CdMAF)

Monolayers (2.5x10⁶ cells in each of 25-cm² flasks) of *Spodoptera frugiperda* (Sf9) cells were co-transfected with cloned plasmid DNA (2 µg) and wild-type (AcMNPV) baculoviral DNA (10 µg) in 950 µl transfection buffer. Recombinant baculovirus plaques were isolated and used for production of the Gc domain III peptide in insect cells. This cloned domain with a molecular weight (MW) of 10,000 and 89 amino acids as shown in Fig. 4, was purified by actin-affinity column (Haddad *et al.*, *Biochemistry* 31:7174, 1992). Two µg of the cloned domain (Cd₁) peptide was treated with immobilized β-galactosidase and sialidase to yield a cloned macrophage activating factor, designated as CdMAF₁.

II. Cloning procedure II: Fusion vector.

1) Cloning the domain responsible for macrophage activation (CdMAF)

A baculovirus fusion vector, pPbac vector (Stratagene, La Jolla, CA), contains human placental alkaline phosphatase secretory signal sequences that direct the nascent cloned peptide toward the secretory pathway of the cells leading to secretion into culture media. The signal sequence is cleaved off by signal-sequence peptidase as the nascent cloned peptide is channeled toward the secretory pathway of the host insect cells leading to secretion of the cloned domain (Cd) peptide. Fig. 5 depicts that the vector carries the stuffer fragment for gene substitution and *lacZ* gene for identification of the gene insertion.

The stuffer fragment of pPbac vector was excised by digesting the vector DNA with restriction enzymes SmaI and BamHI and was removed by electroelution. The E4 cDNA fragment of the Gc protein was digested with HaeIII and BamHI, yielding a fragment practically the same as E4hl (see Fig. 3). This fragment was mixed with the above pPbac vector and ligated with T4 ligase. This strategy not only fixes the orientation of ligation but also fuses the fragment with the reading frame. The *E. coli* DH5aF' cells were transformed with the reaction mixture. The cloned DNA insert was isolated from a number of colonies after digestion with HaeIII and BamHI. The insert was confirmed by sequencing. The sequence confirmed the correct orientation.

2) Isolation of recombinant baculovirus by transfection of Sf9 insect cells with wild type baculovirus and the cloned DNA insert.

For the transfection of insect cells (*Spodoptera frugiperda*, Sf9), linear wild type (AcMNPV) baculoviral DNA and insectin liposomes (Invitrogen, San Diego, CA) have been used. Liposome-mediated transfection of insect cells is the most efficient transfection method available. For transfection to a monolayer of Sf9 cells (2×10^6) in a 60 mm dish, a mixture of the following was gently added:

- 3 μ g cloned plasmid DNA
- 10 μ l linear wild type baculovirus (AcMNPV) DNA (0.1 μ g/ μ l)
- 1 ml medium
- 29 μ l insectin liposomes

The dishes were incubated at room temperature for 4 hours with slow rocking. After transfection, the 1 ml of medium was added and incubated at 27°C in a humidified environment for 48 hours. The resultant transfection lysate was plaque assayed. Purification of recombinant virus, isolation of the cloned domain peptide (Cd₂) and enzymatic generation of the cloned macrophage activating factor designated CdMAF₂ were described as in the Cloning Procedure I. This CdMAF is composed of 94 amino acid residues as shown in Fig. 6, including 9 amino acids from the fusion vector and is referred to herein as CdMAF₂. Although CdMAF₂ has five amino acids more than the CdMAF₁ peptide derived from the non-fusion vector, they exhibited the same biological activities.

**DESCRIPTION OF THE ASSAY METHODS FOR
 α -N-ACETYL GALACTOSAMINIDASE ACTIVITY
IN BLOOD STREAM OF CANCER AND HIV-INFECTED PATIENTS**

1. Assay protocol for detection of α -N-acetylgalactosaminidase in blood stream of cancer and HIV-infected/AIDS patients (Schematic illustration)

Detection procedure for deglycosylating enzyme of serum Gc protein, α -N-acetylgalactosaminidase, in the bloodstream of cancer patients and HIV-infected/AIDS patients.

Step. I. Stepwise 30 and 70% ammonium sulfate precipitation of plasma or serum:

Plasma/sera (1 ml) + 30% and then 70% saturated ammonium sulfate

70% precipitate --> dissolved in 50 mM citrate phosphate buffer (pH 6.0) --> dialyzed against the same buffer at 4°C for overnight.

Step. II. Enzyme assay of α -N-acetylgalactosaminidase

Reaction mixture: 100 μ l of the dialyzed sample + 1.0 ml of 50 mM citrate phosphate buffer (pH 6.0) containing 5 μ moles of p-nitrophenyl N-acetyl- α -D-galactosaminide as substrate.

Incubation time: 60 min, terminated by adding 200 μ l of 0.5 M Na₂CO₃.

Activity measurement: absorbance of amount of released p-nitrophenol at 420 nm and expressed as nmole/mg/min.

2. Enzymatic assay procedure for α -N-acetylgalactosaminidase.

Plasma/serum (1 ml) of a healthy human and patients is precipitated with 70% saturated ammonium sulfate. The ammonium sulfate precipitate is dissolved in 50 mM citrate phosphate buffer (pH 6.0) and dialyzed against the same buffer at 4°C. The volume of the dialysate is made up to 1 ml. Ammonium sulfate precipitation separates the enzyme from inhibitors. The enzyme activity is determined at 37°C in a reaction mixture of 500 μ l containing 50 mM citrate phosphate buffer (pH 6.0) and 5 μ moles of p-nitrophenyl N-acetyl- α -D-galactosaminide as a substrate. The reaction is initiated by addition of 250 μ l of the dialyzed samples and stopped after 60 min by adding 250 μ l of 0.5 M Na₂CO₃ solution. The reaction mixture is centrifuged and amount of released

p-nitrophenol will be determined by the absorbance of the supernatant at 420 nm and expressed as nmole/mg/min.

3. Eenzyme-linked immunosorbent assay (ELISA) for detection of α -N-acetylgalactosaminidase as an antigen.

In the immunoassay procedure for detection of serum or plasma α -N-acetylgalactosaminidase (Nag) as an antigen (NagAg), an antibody-sandwich ELISA kit was prepared.

1) **Preparation of antibody.** Polyclonal antibodies (goat and rabbit) and monoclonal antibodies against α -N-acetylgalactosaminidase (NagAg) were prepared. Immunoglobulin fraction was purified from antisera or monoclonal ascites fluid using ammonium sulfate (50% saturated) fractionation and DE52 ionic exchange or protein A columns.

2) **Conjugation of alkaline phosphatase to antibodies.** Dialyze 5 mg/ml antibody solution in 0.1 M phosphate buffer at pH 6.8 (PBS) overnight at 4°C. Add 100 μ l of dialyzed antibody solution (3 mg/ml) to 90 μ l of 10 mg/ml alkaline phosphatase (immunoassay grade; Boehringer Mannheim, Indianapolis, IN) in a 1.5-ml microcentrifuge tube. Add 5 μ l 25% glutaraldehyde and mix gently. Let stand at room temperature. Remove 25- μ l samples at time 0, 5, 10, 15, 30, 60 and 120 min and place a separate 1.5-ml microcentrifuge tubes. Add 125 μ l PBS to each sample, then add 1.1 ml Tris/ovoalbumin solution. Dialyze samples against PBS overnight at 4°C. Test each sample for alkaline phosphatase activity using a direct ELISA to determine which conjugation time yields the most active enzyme conjugation. A 30 min conjugation time yielded the best enzyme activity. This optimal conjugation time was used for preparation of a larger amount of alkaline phosphatase-antibody conjugates.

3) **Preparation of antibody coated microtiter plates.** Using multichannel pipets and tips, dispense 50 μ l of immunoglobulin solution 2 to 10 μ g/ml in PBSN (PBS containing 0.05% sodium azide) into each well of a microtiter plate (microwell). Wrap the plates in plastic wrap to seal and incubate 2 hrs at 37°C or overnight at room temperature. Rinse the coated plate by flooding with distilled water more than three times. Fill each well with blocking buffer (PBS containing 0.05% tween 20, 1 mM EDTA, 0.25% BSA and 0.05% NaN_3) dispensed as a stream from a bottle and incubate

30 min at room temperature. Rinse the plate three times with distilled water and remove the residual liquid by gently flicking it face down onto paper towels.

4) Antigen (NagAg) preparation.

(a) **Standard antigen for standard curve.** Prepare a standard antigen-dilution series by successive 1:3 dilutions of antigen stock in blocking buffer. The range of dilution spans from 0.1 to 1000 ng/ml.

(b) **Test antigen.** Dilute the serum/plasma of cancer or HIV-infected patients 10- to 100-fold because they contain 20 to 1000 ng of apoprotein/ml of patient serum/plasma.

5) Assay:

Step I. Add 50- μ l aliquots of the antigen test solutions and the standard antigen dilutions to the antibody coated wells and incubate 2 hrs at room temperature. Rinse the plate three times in distilled water. Fill each well with blocking buffer and incubate 10 min at room temperature. Rinse three times with water and remove the residual liquid.

Step II. Add 50 μ l specific antibody-alkaline phosphatase conjugate (typically 25 to 400 ng/ml) and incubate 2 hr at room temperature. Wash the plate as in Step I.

Step III. Add 75 μ l of p-nitrophenyl phosphate (NPP) substrate solution to each well and incubate 1 hr at room temperature. Enzyme activity causes the solution to change color. Color intensity relates to the presence or absence of NagAg. Read the plate on a microtiter plate reader with a 405-nm filter.

6) Data analysis. Prepare a standard curve constructed from the data produced by serial dilutions of the standard antigen. Plot the antigen concentration on the x axis which is a log scale, and absorbance on the y axis which is a linear scale. Interpolate the concentration of antigen (NagAg) in the test solution. Calculate the α -N-acetylgalactosaminidase activity. Since antibodies to cancer and HIV enzymes are specific to the respective enzymes (NagAg), this antibody sandwich ELISA distinguishes the individual enzyme as well as the antigen (α -galactosidase) in the control. Serum/plasma α -N-acetylgalactosaminidase activity was also determined by interpolating the NagAg concentration in another standard curve prepared for the α -N-acetylgalactosaminidase activity vs. NagAG concentration. This ELISA assay for the enzyme activity is not only accurate but also economical.

SUPPORTING OBSERVATIONS

A. Effects of Cloned Macrophage Activating Factors, GcMAFc and CdMAF on Cultured Phagocytes (Macrophages and Osteoclasts).

The three hour treatment of human macrophages and osteoclasts with picogram quantities (pg) of the cloned macrophage activating factors, GcMAFc and CdMAF, resulted in a greatly enhanced superoxide generating capacity of these phagocytes as shown in Table 1. The levels of the phagocyte activation are similar to those of macrophage activation by GcMAF (Yamamoto *et al.*, AIDS Res. Human Ret. 11:1373, 1995).

Table 1. Activation of phagocytes by *in vitro* treatment with GcMAF and its cloned derivatives.

	Conc. pg/ml	<u>nmole of superoxide produced/min/10⁶ phagocytes</u>		
		<u>Human macrophages*</u>	<u>Mouse peritoneal macrophages</u>	<u>Human osteoclasts</u>
GcMAFc	0	0.07	0.06	0.03
	10	3.20	3.46	2.56
	100	5.18	5.08	4.22
CdMAF	0	0.01	0.02	0.08
	10	2.96	2.87	2.43
	100	4.26	4.53	4.09

*Peripheral blood monocytes/macrophages of cancer patients. Similar results were also observed when those of HIV-infected patients were used.

B. Activation of Mouse Peritoneal Macrophages by Administration of Cloned Macrophage Activating Factors, GcMAFc and CdMAF.

One day post-administration of a picogram quantity (10 and 100 pg/mouse) of GcMAFc or CdMAF to BALB/c mice, peritoneal macrophages were isolated and assayed for superoxide generating capacity. As shown in Table 2, the macrophages were efficiently activated. These results are similar to those of macrophage activation with GcMAF (Naraparaju and Yamamoto, Immunol. Lett. 43:143, 1994).

Table 2. Activation of mouse peritoneal macrophages by administration of cloned GcMAF derivatives.

	<u>Dosage</u> <u>pg/mouse</u>	<u>nmole of superoxide produced/min/10⁶ phagocytes</u> <u>Mouse peritoneal macrophages</u>
GcMAFc	0	0.05
	10	3.18
	100	5.23
CdMAF	0	0.03
	10	2.54
	100	4.23

C. Therapeutic Effects of GcMAF, GcMAFc or CdMAF on Tumor Bearing Mice and Osteopetrotic Mice.

1) Therapeutic effects of GcMAF, GcMAFc or CdMAF on Ehrlich ascites tumor bearing mice.

When BALB/c mice were administered with GcMAF, GcMAFc or CdMAF (100 pg/mouse) and received 10⁵ Ehrlich ascites tumor cells/mouse, they survived for at least 5 weeks. All the control mice received only the ascites tumor and died in approximately 2 weeks. When mice were administered with an additional GcMAF, GcMAFc or CdMAF (100 pg/mouse) 4 days post-transplantation, the tumor cells were completely eliminated (Table 3).

Table 3. Therapeutic effects of GcMAF and cloned derivatives on mice bearing Ehrlich ascites tumor.

<u>Group</u>	<u>No. of mice</u>	<u>Post-transplantation treatment^a</u>	<u>No. of mice survived/period</u>
Group 1.	6 mice	untreated control	6 mice/13 ± 3 days
	10 mice	day 0 100 pg GcMAF/mouse	10 mice/36 ± 7 days
Group 2.	6 mice	untreated control	6 mice/14 ± 4 days
	10 mice	day 0 100 pg GcMAFc/mouse	10 mice/35 ± 6 days

Group 3.

6 mice	untreated control	6 mice/14 \pm 5 days
10 mice	day 0 100 pg CdMAF/mouse	10 mice/34 \pm 3 days

Group 4.

8 mice	untreated control	8 mice/15 \pm 5 days
12 mice	day 0 100 pg GcMAF/mouse	
day 4	100 pg GcMAF/mouse	12 mice/ >65 days

Group 5.

8 mice	untreated control	8 mice/14 \pm 5 days
12 mice	day 0 100 pg GcMAFc/mouse	
	day 4 100 pg GcMAFc/mouse	12 mice/ >65 days

Group 6.

8 mice	untreated control	8 mice/14 \pm 5 days
12 mice	day 0 100 pg CdMAF/mouse	
	day 4 100 pg CdMAF/mouse	12 mice/ >65 days

^aGcMAF, GcMAFc and CdMAF were administered intramuscularly (systemically) and mice in all groups receive 10^5 tumor cells/mouse.

2) Therapeutic effects of GcMAF and cloned GcMAF derivatives (GcMAFc and CdMAF) on osteopetrotic mice.

The administration of GcMAFc or CdMAF to new born litters of osteopetrotic op/op mouse was performed by the weekly injection of 100 picograms for four weeks beginning from a day after birth. Mice were sacrificed at 28 days. The tibiae were removed from the treated and untreated control mice, longitudinally bisected, and examined under a dissecting microscope to measure the size of the bone marrow cavity. The cavity size was expressed as a percentage of the distance between the epiphyseal plates of the tibia. The untreated mouse group formed bone marrow with 30% of the total length of tibia. The treated mouse group experienced a 20% increased bone marrow formation over that of the untreated mouse group. This increased bone marrow cavity formation is an indication of osteoclast activation and increased osteoclastic bone resorption.

D. The Origin of Immunosuppression in Cancer and HIV-infected Patients.

The source of the plasma α -N-acetylgalactosaminidase in cancer patients appeared to be cancerous cells. High α -N-acetylgalactosaminidase activities were detected in tumor tissue homogenates of various organs, including eleven different tumor tissues including 4 lung, 3 breast, 3 colon and 1 cervix tumors, though the α -N-acetylgalactosaminidase activity varied from 15.9 to 50.8 nmoles/mg/min. Surgical removal of malignant lesions in human cancer results in the subtle decrease of plasma α -N-acetylgalactosaminidase activity with the concomitant increase in the precursor activity, particularly if malignant cells are localized.

In a preclinical mouse tumor model, BALB/c mice were transplanted with 5×10^5 Ehrlich ascites tumor cells/mice into peritoneal cavity and analyzed for serum α -N-acetylgalactosaminidase activity. When serum enzyme levels were measured as transplanted Ehrlich ascites tumor grew in the mouse peritoneal cavity, the enzyme activity was directly proportional to the tumor burden (the total cell counts in peritoneal cavity) as shown in Fig. 7. This was also confirmed with nude mice transplanted with KB cells (human oral squamous cell carcinoma cell line). Serum α -N-acetylgalactosaminidase activity increased as tumor size (measured by weight) of the solid tumor increased. Thus, I have been using plasma α -N-acetylgalactosaminidase activity as a prognostic index to monitor the progress of therapy.

Radiation therapy of human cancer decreased plasma α -N-acetylgalactosaminidase activity with a concomitant increase of precursor activity. This implies that radiation therapy decreases the number of cancerous cells capable of secreting α -N-acetylgalactosaminidase. These results also confirmed that plasma α -N-acetylgalactosaminidase activity has an inverse correlation with the MAF precursor activity of Gc protein. Even after surgical removal of tumor lesions in cancer patients, most post-operative patients carried significant amounts of α -N-acetylgalactosaminidase activity in their blood stream. The remnant cancerous lesions in these post-operative patients cannot be detectable by any other procedures, e.g., X-ray, scintigraphy, etc. I have been using this most sensitive enzyme assay as a prognostic index during the course of GcMAF therapy.

HIV-infected cells appeared to secrete α -N-acetylgalactosaminidase into the blood stream. When peripheral blood mononuclear cells (PBMC) of HIV-infected patients

were cultured and treated with mitomycin as a provirus inducing agent (Sato *et al.*, Arch. Virol. 54:333, 1977), α -N-acetylgalactosaminidase was secreted into the culture media. These results led me to suggest that α -N-acetylgalactosaminidase is a virus coded product. In fact, HIV-envelope protein gp120 appears to carry the α -N-acetylgalactosaminidase activity.

E. Therapeutic Effects of GcMAF, GcMAFc and CdMAF on Human Cancer and Virus Infected Patients.

1. Cancer patients: Therapeutic effect of GcMAF on prostate, breast and colon cancer and adult leukemia patients.

The administration of GcMAF (100 and 500 ng/human) to healthy volunteers resulted in the greatly enhanced activation of macrophages as measured by the 7-fold enhanced phagocytic capacity and the 15-fold superoxide generating capacity of macrophages. The administration of GcMAF showed no signs of any side effects to the recipients. Administration of various doses (100 pg to 10 ng/mouse) to a number of mice produced neither ill effects nor histological changes in various organs including liver, lung, kidney, spleen, brain, etc. When patients with various types of cancer were treated with GcMAF (100 ng/week), remarkable curative effects on various types of cancer were observed. The therapeutic efficacy of GcMAF on patients bearing various types of cancers was assessed by tumor specific serum α -N-acetylgalactosaminidase activity because the serum enzyme level is proportional to the total amount of cancerous cells (tumor burden). Curative effects of GcMAF on prostate, breast and colon cancer are illustrated in Figs. 8a to 8c. After 25 weekly administrations of 100 ng GcMAF the majority (>90%) of prostate and breast cancer patients exhibited insignificantly low levels of the serum enzyme. A similar result was also observed after 35 GcMAF administrations to colon cancer patients. Similar curative effects of GcMAF on lung, liver, stomach, brain, bladder, kidney, uterus, ovarian, larynx, esophagus, oral and skin cancers were observed. Thus, GcMAF appeared to be effective on a variety of cancers indiscriminately. However, GcMAF showed no evidence of side effects in patients after more than 6 months of therapy. This was also confirmed by blood cell count profile, liver and kidney functions, etc.

When GcMAFc (100 ng/week) and CdMAF (100 ng/week) were administered to two prostate cancer patients each, curative effects similar to those of GcMAF were observed.

2. Virus infected patients

Treatment of peripheral blood macrophages of HIV-infected/AIDS patients with 100 pg GcMAF/ml resulted in a greatly enhanced macrophage activation (Yamamoto *et al.*, AIDS Res. Human Ret. 11:1373, 1995). HIV-infected patients carry anti-HIV antibodies. HIV-infected cells express the viral antigens on the cell surface. Thus, macrophages have a potential to eliminate the infected cells via Fc-receptor mediated cell-killing/ingestion when activated.

Similarly, treatment of peripheral blood macrophages of patients chronically infected with Epstein-Barr virus (EBV) and with herpes zoster with 100 ng GcMAF/ml resulted in a greatly enhanced macrophage activation. Like HIV, EBV infects lymphocytes (B cells). Since these enveloped viruses code for α -N-acetylgalactosaminidase and infected cells secrete it into blood stream, this enzyme activity in patient sera can be used as a prognostic index during therapy. After approximately 25 administrations of GcMAF (100 ng/week) to patients chronically infected with EBV and with herpes zoster, the enzyme activity decreased to that of healthy control levels. When GcMAFc (100 ng/week) and CdMAF (100 ng/week) were administered to EBV-infected patients, curative effects similar to those of GcMAF were observed.

F. Adjuvant Activities of GcMAF, GcMAFc and CdMAF for Immunization and Vaccinations: Rapid increase of the number of antibody secreting cells (PFC) in mice after administration of GcMAF and sheep erythrocytes.

BALB/c mice were inoculated with SRBC 6 hours after the intraperitoneal administration of 50 pg GcMAF/mouse. At various intervals (1-5 days) after immunization, IgM-antibody secreting cells in the spleen were determined using the Jerne plaque assay (Jerne *et al.*, Cell-bound antibodies, Wistar Institute Press, 1963). One day post-administration of GcMAF and SRBC produced 1.35×10^4 PFC/spleen. Two days after administration of GcMAF and SRBC, the number of antibody secreting cells had increased to 8.2×10^4 PFC/spleen. By the 4th day the number of antibody secreting cells reached the maximal level (about 23.6×10^4 PFC/spleen), as shown in

Table 4. In contrast, mice that received an injection of SRBC alone produced about 3.8×10^4 PFC/spleen, 4 days after SRBC-injection.

To ascertain the dose response, mice were injected with SRBC 6 hours after administration of various doses of GcMAF ranging from 1 to 50 pg/mouse. On the 4th day post-administration of GcMAF and SRBC, the number of antibody secreting cells per spleen was determined by the Jerne plaque assay. On the 4th day post-administration there was a commensurate increase in the number of plaque forming cells as the concentration of GcMAF was increased above 1 pg per mouse. At a GcMAF dose of 5, 10 and 50 pg/mouse, I detected 12.6×10^4 , 20.2×10^4 and 24.3×10^4 PFC/spleen, respectively. Therefore, GcMAF is a potent adjuvant for immunization.

Table 4. Time course studies on development of cells secreting antibody against sheep erythrocytes (SRBC) in BALB/c mice after administration of GcMAF and SRBC^a.

After SRBC immunization (days)	<u>Antibody secreting cells/spleen ($\times 10^4$)</u>	
	SRBC only	GcMAF + SRBC
1	0.01 ± 0.002	1.35 ± 0.21
2	0.08 ± 0.02	8.28 ± 1.26
3	1.18 ± 0.42	14.42 ± 2.32
4	3.86 ± 0.95	23.68 ± 6.05
5	2.15 ± 0.63	18.63 ± 3.43

^aMice were inoculated with SRBC (10^8 cells) 6 hr after administration of GcMAF (50 pg/mouse). The number of plaques (IgM secreting cells) was quantified microscopically on various days post-SRBC injection. The number of plaque-forming cells (PFC) per spleen is expressed as the mean value of triplicate assays \pm SEM.

Without further elaboration the foregoing will so fully illustrate my invention that others may, by applying current or future knowledge, adapt the same for use under various conditions of service.

References Cited

The following references are cited and their entire text is incorporated fully herein as are all references set forth above in the specification.

U.S. PATENT DOCUMENTS

U.S. Patent Nos. 5,177,001, 5,177,002 and 5,326,749 (Yamamoto).

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CLAIMS

I claim:

1. A process for cloning vitamin D₃-binding protein (Gc protein) into baculovirus comprising the step of selecting and using a baculovirus vector to clone the vitamin D₃-binding protein Gc protein (Gc protein):

- (a) isolating the full length Gc protein cDNA library from human liver λ gt11 by the use of immunoscreening method and inserting into non-fusions baculovirus vector;
- (b) isolating recombinant baculovirus carrying the full length Gc protein cDNA by cotransfecting the insect cells (Sf9) with vector DNA and wild type baculovirus DNA; and
- (c) propagating the recombinant baculovirus in insect cells and purifying the cloned Gc protein.

2. A process for producing a cloned macrophage activating factor (GcMAFc) comprising contacting cloned Gc protein in vitro with immobilized β -galactosidase and sialidase and obtaining the cloned macrophage activating factor (GcMAFc).

3. A process for cloning vitamin D₃-binding protein domain III (Gc domain III) into baculovirus comprising the step of selecting and utilizing a baculovirus vector to clone the vitamin D₃-binding protein domain III (Gc domain III):

- (a) isolating an initiation-leader sequence plus 4 amino acids of the 5' terminal end of the Gc protein cDNA and the Gc protein domain III cDNA by digestion of the Gc protein cDNA with EcoRI and HaeIII enzymes and inserting into the EcoRI site of a non-fusions baculovirus vector (pVL1393) by the use of ligase; or isolating the Gc protein domain III cDNA using HaeIII and BamHI enzymes and inserting into a fusion baculovirus vector pPbac by substituting a SmaI-BamHI stuffer fragment downstream of the signal sequence by the use of ligase;
- (b) isolating the recombinant baculovirus carrying the domain III of Gc protein cDNA by cotransfecting the insect cells (Sf9) with vector DNA and wild type baculovirus DNA; and
- (c) propagating the recombinant baculovirus in the insect cells and purification of the cloned domain protein (Cd).

4. A process for producing a cloned macrophage activating factor (CdMAF) comprising contacting cloned Gc domain III in vitro with immobilized β -galactosidase and sialidase and obtaining the macrophage activating factor (CdMAF).
5. Use of a Gc protein macrophage activating factor (GcMAF), the GcMAF being a product of contacting serum Gc protein in vitro with immobilized β -galactosidase and sialidase, for treating a person suffering from cancer.
6. Use of a cloned macrophage activating factor (GcMAFc), which is a product of the process according to claim 2, for treating a person suffering from cancer.
7. Use of a cloned macrophage activating factor (CdMAF), which is a product of the process according to claim 4, for treating a person suffering from cancer.
8. Use of a macrophage activating factor (GcMAF), which is a product of contacting serum Gc protein in vitro with immobilized β -galactosidase and sialidase, for treating a person suffering from human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) or herpes zoster.
9. Use of a macrophage activating factor (GcMAFc), which is a product of the process according to claim 2, for treating a person suffering from human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) or herpes zoster.
10. Use of a macrophage activating factor (CdMAF), which is a product of the process according to claim 4, for treating a person suffering from human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) or herpes zoster.
11. A macrophage activating factor (GcMAFc), which is a product of the process according to claim 2.
12. A macrophage activating factor (CdMAF), which is a product of the process according to claim 4.
13. Use of a macrophage activating factor (GcMAFc), which is a product of the process according to claim 2, for promoting bone marrow formation in osteopetrotic patients.
14. Use of a macrophage activating factor (CdMAF), which is a product of the process according to claim 4, for promoting bone marrow formation in osteopetrotic patients.

15. An adjuvant comprising a macrophage activating factor (GcMAF) which is a product of contacting serum Gc protein in vitro with immobilized β -galactosidase and sialidase.

16. An adjuvant comprising a macrophage activating factor (GcMAFc), which is a product of the process according to claim 2.

17. An adjuvant comprising a macrophage activating factor (CdMAF), which is a product of the process according to claim 4.

18. A cloned vitamin D₃-binding protein (Gc protein) having an amino acid sequence of Fig. 2 (SEQ. ID. NO:1)(GcMAFc).

19. A cloned vitamin D₃-binding protein domain III (Gc domain III) having an amino acid sequence of Fig. 4 (SEQ ID. NO:2)(CdMAF₁).

20. A cloned vitamin D₃-binding protein domain III (Gc domain III) having an amino acid sequence of Fig. 6 (SEQ ID. NO:3)(CdMAF₂).

21. A process for determining α -N-acetylgalactosaminidase activity in plasma or serum of cancer patients and patients suspected of having cancer comprising the steps of ammonium sulfate precipitation of plasma or serum containing α -N-acetylgalactosaminidase activity to produce a precipitate, dialysis of the precipitate with citrate phosphate buffer and then incubation of the precipitate with p-nitrophenyl-N-acetyl- α -D-galactosaminide.

22. A process for determining α -N-acetylgalactosaminidase activity in plasma or serum of patients and persons suspected of carrying human immunodeficiency virus comprising the steps of ammonium sulfate precipitation of plasma or serum containing α -N-acetylgalactosaminidase activity to produce a precipitate, dialysis of the precipitate with citrate phosphate buffer and then incubation of the precipitate with p-nitrophenyl-N-acetyl- α -D-galactosaminide.

23. An enzyme-linked immunosorbent assay (ELISA) process for determining α -N-acetylgalactosaminidase activity as an antigen in plasma or serum of cancer patients and patients suspected of having cancer comprising the steps of preparing a kit consisting of polyclonal and monoclonal antibodies against α -N-acetylgalactosaminidase, diluted serum or plasma containing α -N-acetylgalactosaminidase (as antigen), an antibody captured microtiter plate and alkaline phosphatase-antibody conjugates and antibody-sandwich ELISA assay by stepwise incubation of a microtiter plate with diluted serum

or plasma, alkaline phosphatase-linked antibody conjugates and p-nitrophenyl-phosphate as the substrate.

24. An enzyme-linked immunosorbent assay (ELISA) process for determining α -N-acetylgalactosaminidase activity in the plasma or serum of patients and persons suspected of carrying human immunodeficiency virus comprising the steps of preparing a kit consisting of polyclonal and monoclonal antibodies against α -N-acetylgalactosaminidase, diluted serum or plasma containing α -N-acetylgalactosaminidase (as antigen), antibody captured microtiter plate and alkaline phosphatase-antibody conjugates and antibody-sandwich ELISA assay by stepwise the incubation of microtiter plate with diluted serum or plasma, alkaline phosphatase-linked antibody conjugates and p-nitrophenyl-phosphate as the substrate.

1/10

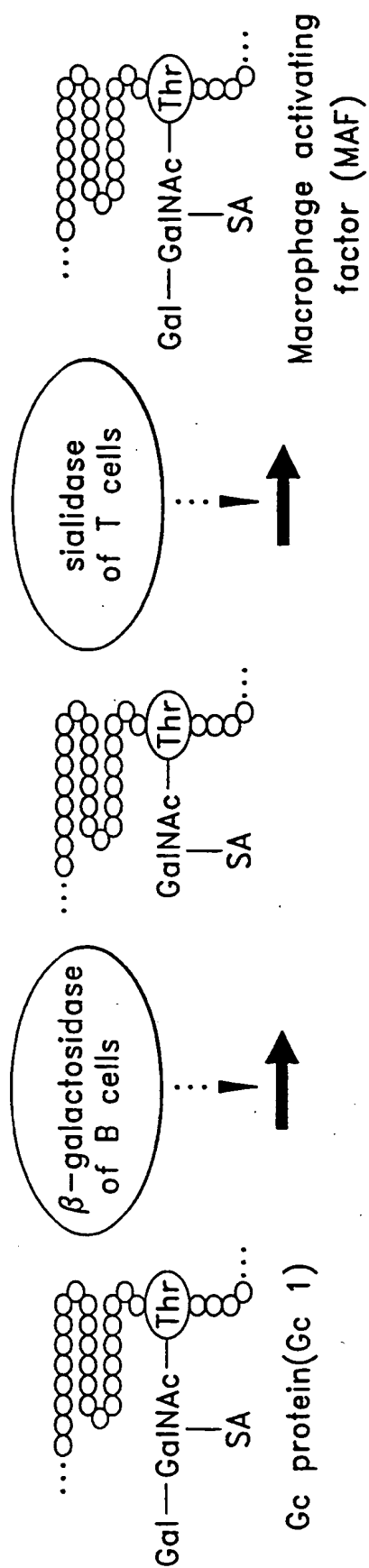


FIG. 1A

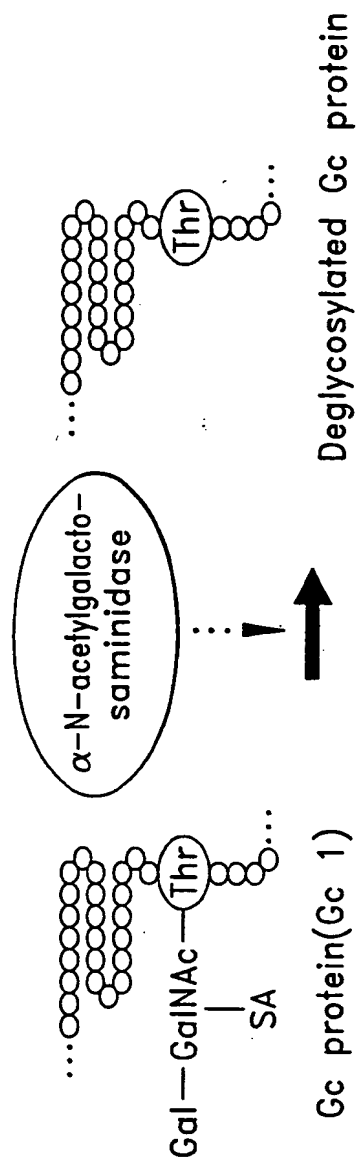


FIG. 1B

SUBSTITUTE SHEET (RULE 26)

2/10

Leu	Glu	Arg	Gly	Arg	Asp	Tyr	Glu	Lys	Asn	Lys	Val	Cys	Lys	Glu	Phe	Ser	His	Leu	Gly	10	20
Lys	Glu	Asp	phe	Thr	Ser	Leu	Ser	Leu	Val	Leu	Tyr	Ser	Arg	Lys	Phe	Pro	Ser	Gly	Thr	30	40
Phe	Glu	Gln	Val	Ser	Gln	Leu	Val	Lys	Glu	Val	Val	Ser	Leu	Thr	Glu	Ala	Cys	Cys	Ala	50	60
Glu	Gly	Ala	Asp	Pro	Asp	Cys	Tyr	Asp	Thr	Arg	Thr	Ser	Ala	Leu	Ser	Ala	Lys	Ser	Cys	70	80
Glu	Ser	Asn	Ser	Pro	Phe	Pro	Val	His	Pro	Gly	Thr	Ala	Glu	Cys	Cys	Thr	Lys	Glu	Gly	90	100
Leu	Glu	Arg	Lys	Leu	Cys	Met	Ala	Ala	Leu	Lys	His	Gln	Pro	Gln	Glu	Phe	Pro	Thr	Tyr	110	120
Val	Glu	Pro	Thr	Asn	Asp	Glu	Ile	Cys	Glu	Ala	Phe	Arg	Lys	Asp	Pro	Lys	Glu	Tyr	Ala	130	140
Asn	Gln	Phe	Met	Trp	Glu	Tyr	Ser	Thr	Asn	Tyr	Glu	Gln	Ala	Pro	Leu	Ser	Leu	Leu	Val	150	160
Ser	Tyr	Thr	Lys	Ser	Tyr	Leu	Ser	Met	Val	Gly	Ser	Cys	Cys	Thr	Ser	Ala	Ser	Pro	Thr	170	180
Val	Cys	Phe	Leu	Lys	Glu	Arg	Leu	Gln	Leu	Lys	His	Leu	Ser	Leu	Leu	Thr	Thr	Leu	Ser	190	200
Asn	Arg	Val	Cys	Ser	Gln	Tyr	Ala	Ala	Tyr	Gly	Glu	Lys	Lys	Ser	Arg	Leu	Ser	Asn	Leu	210	220
Ile	Lys	Leu	Ala	Gln	Lys	Val	Pro	Thr	Ala	Asp	Leu	Glu	Asp	Val	Leu	Pro	Leu	Ala	Glu	230	240
Asp	Ile	Thr	Asn	Ile	Leu	Ser	Lys	Cys	Cys	Glu	Ser	Ala	Ser	Glu	Asp	Cys	Met	Ala	Lys	250	260
Glu	Leu	Pro	Glu	His	Thr	Val	Lys	Leu	Cys	Asp	Asn	Leu	Ser	Thr	Lys	Asn	Ser	Lys	Phe	270	280
Glu	Asp	Cys	Cys	Gln	Glu	Lys	Thr	Ala	Met	Asp	Val	Phe	Val	Cys	Thr	Tyr	Phe	Met	Pro	290	300
Ala	Ala	Gln	Leu	Pro	Glu	Leu	Pro	Asp	Val	Arg	Leu	Pro	Thr	Asn	Lys	Asp	Val	Cys	Asp	310	320
Pro	Gly	Asn	Thr	Lys	Val	Met	Asp	Lys	Tyr	Thr	Phe	Glu	Leu	Ser	Arg	Arg	Thr	His	Leu	330	340
Pro	Glu	Val	Phe	Leu	Ser	Lys	Val	Leu	Glu	Pro	Thr	Leu	Lys	Ser	Leu	Gly	Glu	Cys	Cys	350	360
Asp	Val	Glu	Asp	Ser	Thr	Thr	Cys	phe	Asn	Ala	Lys	Gly	Pro	Leu	Leu	Lys	Lys	Glu	Leu	370	380
Ser	Ser	Phe	Ile	Asp	Lys	Gly	Gln	Glu	Leu	Cys	Ala	Asp	Tyr	Ser	Glu	Asn	Thr	Phe	Thr	390	400
Glu	Tyr	Lys	Lys	Lys	Leu	Ala	Glu	Arg	Leu	Lys	Ala	Lys	Leu	Pro	Glu	Ala	Thr	Pro	Thr	410	420
Glu	Leu	Ala	Lys	Leu	Val	Asn	Lys	Arg	Ser	Asp	Phe	Ala	Ser	Asn	Cys	Cys	Ser	Ile	Asn	430	440
Ser	Pro	Pro	Leu	Tyr	Cys	Asp	Ser	Glu	Ile	Asp	Ala	Glu	Leu	Lys	Asn	Ile	Leu			450	458

FIG. 2**SUBSTITUTE SHEET (RULE 26)**

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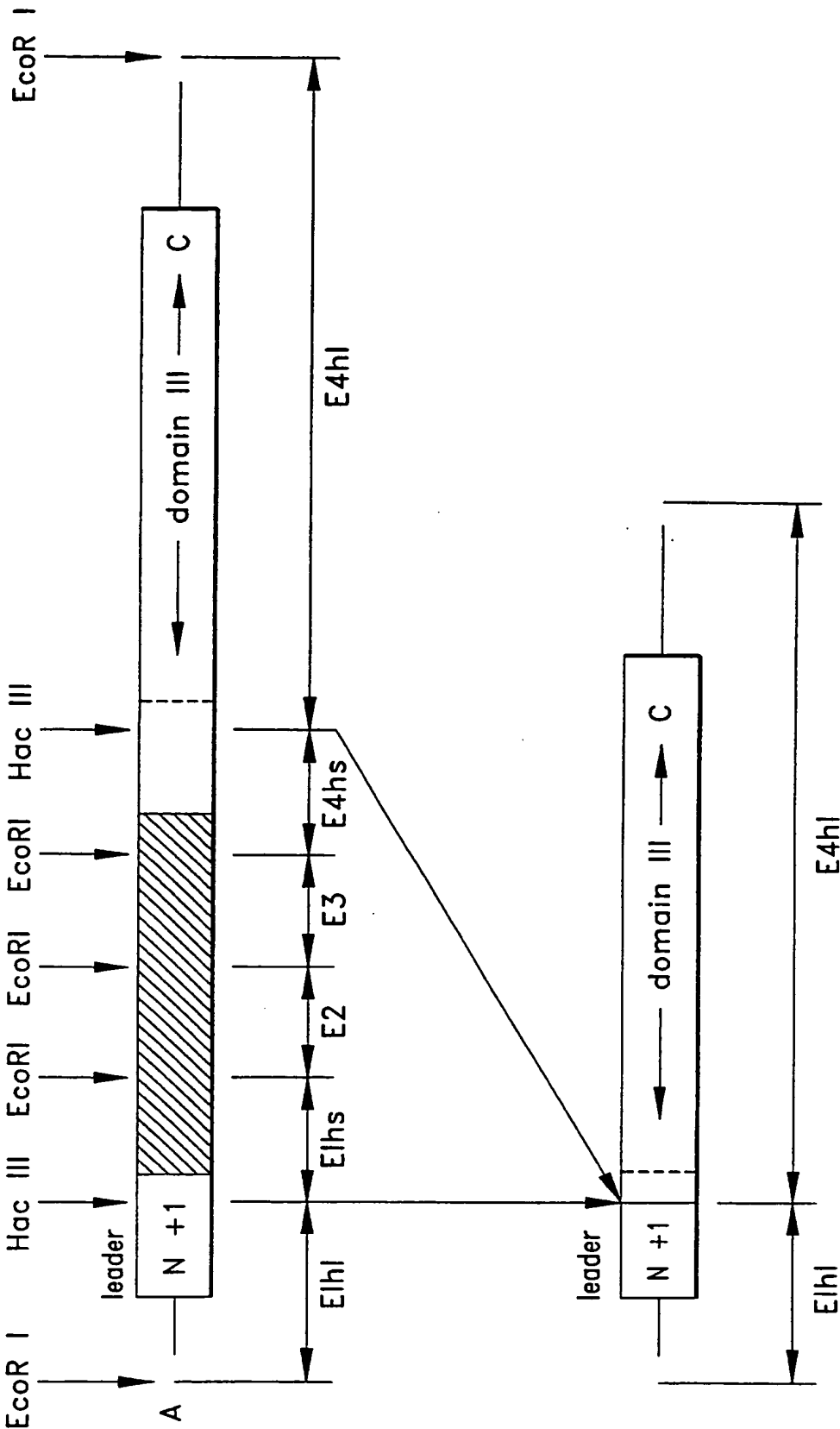


FIG. 3

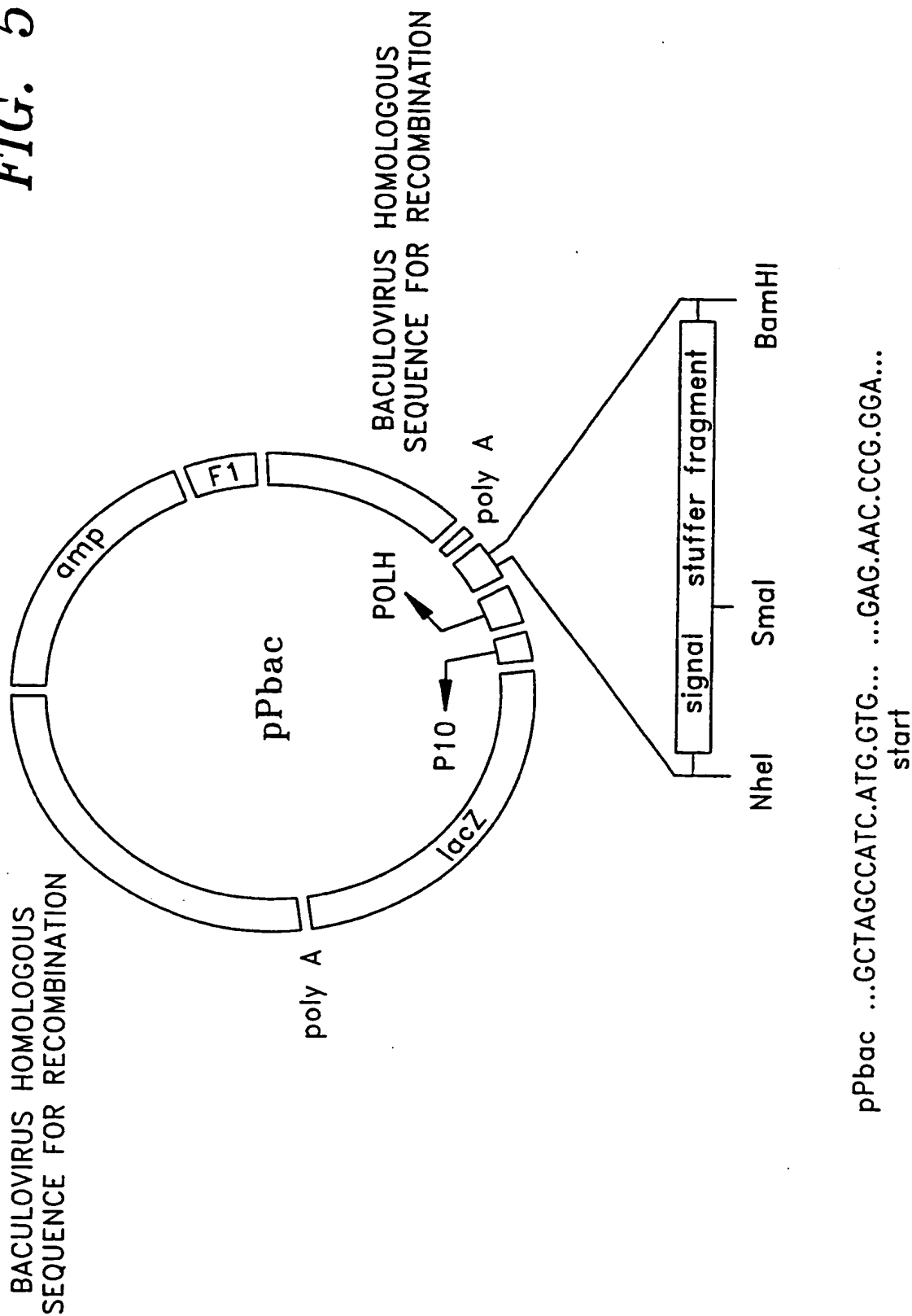
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Leu	Glu	Arg	Gly	Pro	Leu	Leu	Lys	Lys	Glu	Leu	Ser	Ser	Phe	Ile	Asp	Lys	Gly	Gln	Glu
									10										20
Leu	Cys	Ala	Asp	Tyr	Ser	Glu	Asn	Thr	Phe	Thr	Glu	Tyr	Lys	Lys	Lys	Leu	Ala	Glu	Arg
									30										40
Leu	Lys	Ala	Lys	Leu	Pro	Glu	Ala	Thr	Pro	Thr	Glu	Leu	Ala	Lys	Leu	Val	Asn	Lys	Arg
									50										60
Ser	Asp	Phe	Ala	Ser	Asn	Cys	Cys	Ser	Ile	Asn	Ser	Pro	Pro	Leu	Tyr	Cys	Asp	Ser	Glu
									70										80
Ile	Asp	Ala	Glu	Leu	Lys	Asn	Ile	Leu											
									89										

FIG. 4

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FIG. 5



SUBSTITUTE SHEET (RULE 26)

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Ile Ile Pro Val Glu Glu Glu Asn Pro Pro¹⁰ Leu Leu Lys Lys Glu Leu Ser Ser Phe²⁰ Ile⁴⁰
Asp Lys Gly Gln Glu Leu Cys Ala Asp Tyr³⁰ Ser Glu Asn Thr Phe Thr Glu Tyr Lys Lys⁶⁰
Lys Leu Ala Glu Arg Leu Lys Ala Lys Leu⁵⁰ Pro Glu Ala Thr Pro Thr Glu Leu Ala Lys⁸⁰
Leu Val Asn Lys Arg Ser Asp Phe Ala Ser Asn Cys Cys Ser Ile Asn Ser Pro Pro Leu⁹⁰
Tyr Cys Asp Ser Glu Ile Asp Ala Glu Leu Lys Asn Ile Leu⁹⁴

FIG. 6

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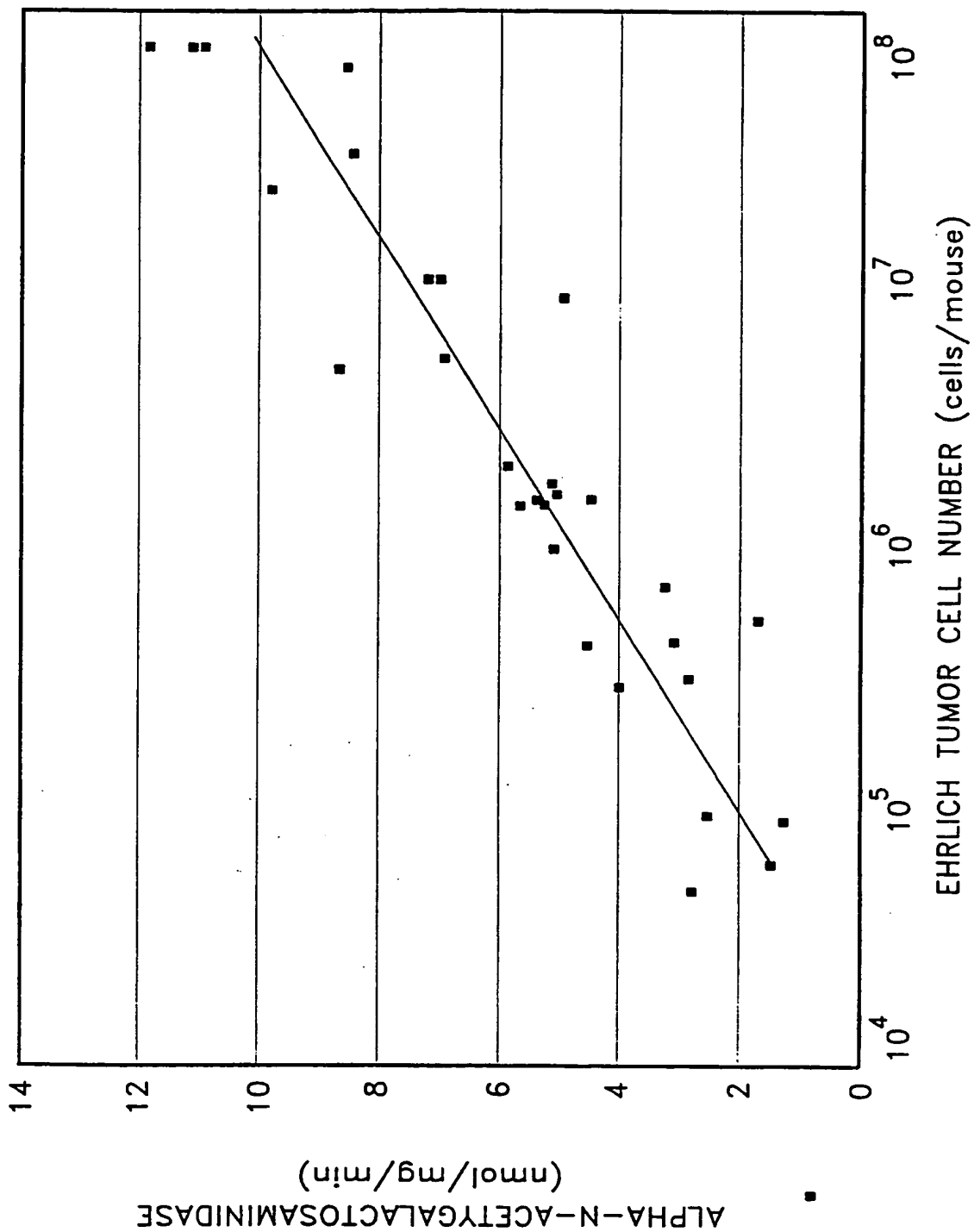
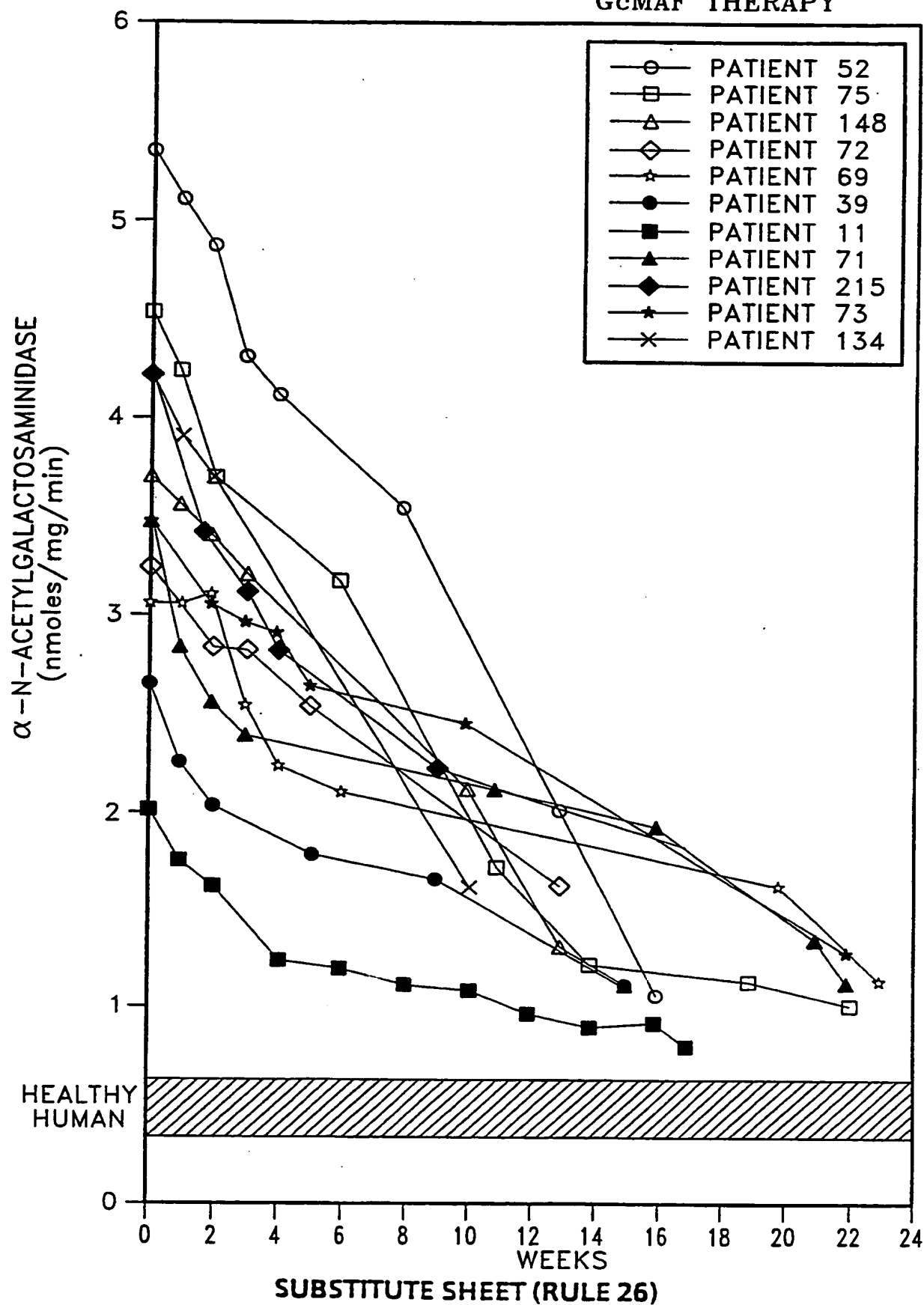


FIG. 7

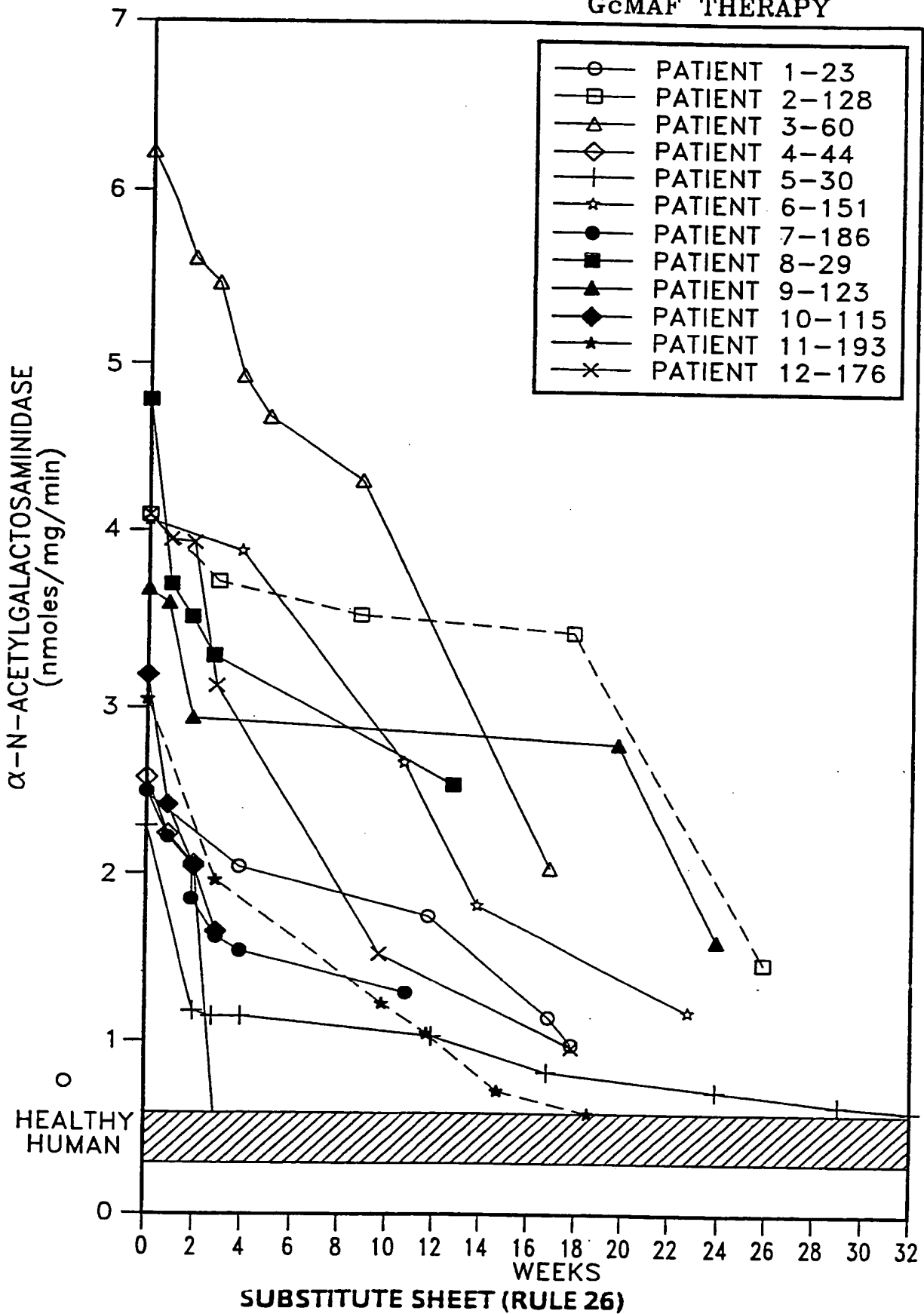
SUBSTITUTE SHEET (RULE 26)

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FIG. 8A**PROSTATE CANCER
GcMAF THERAPY**

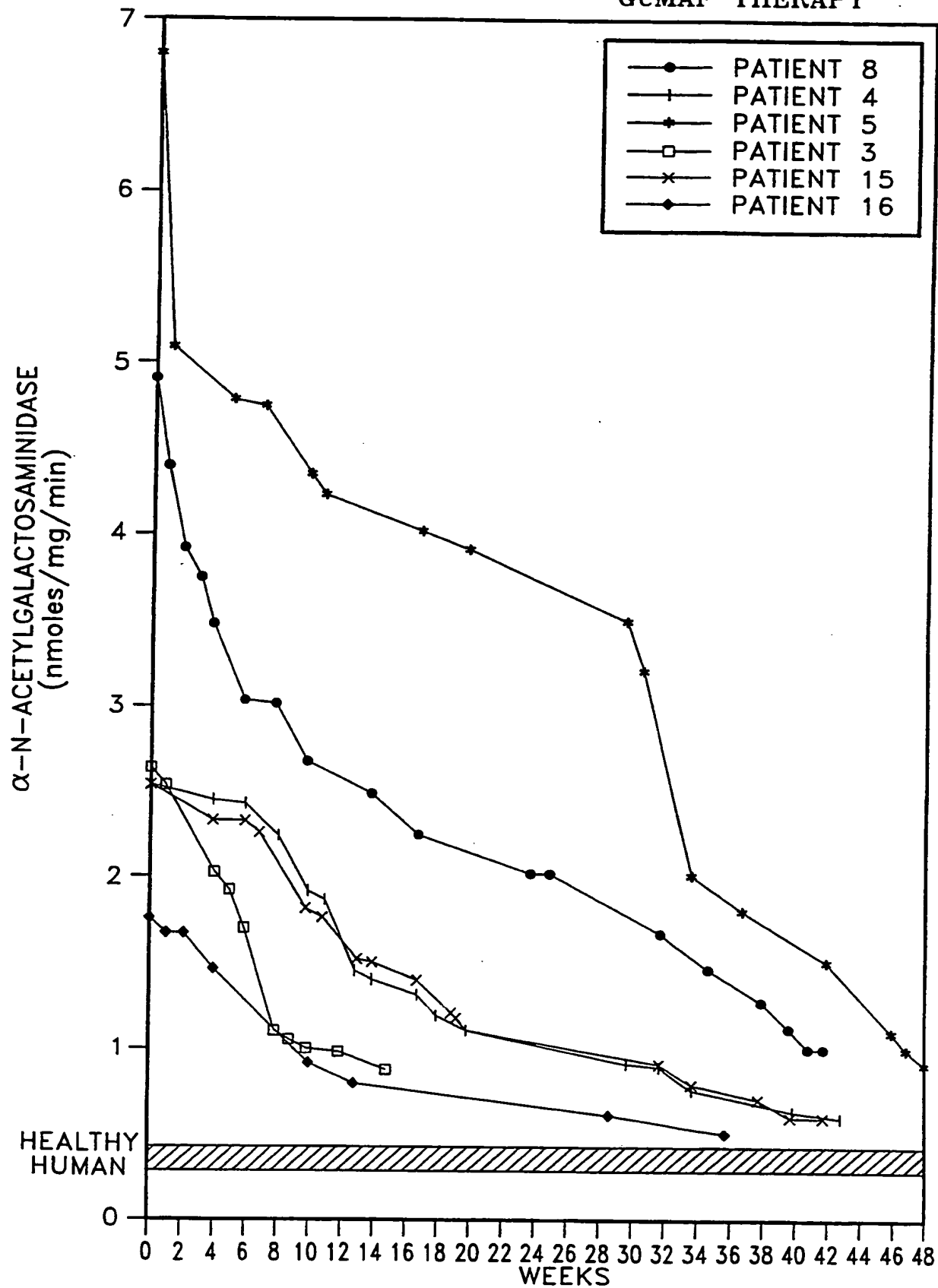
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FIG. 8B**BREAST CANCER
GcMAF THERAPY**

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FIG. 8C

COLON CANCER
GcMAF THERAPY

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/08867

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/10; C07K 14/435

US CL : 435/172.1, 172.2, 172.3, 320.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.1, 172.2, 172.3, 320.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	GENOMICS, Volume 16, issued 1993, Witke et al, "Complete Structure of the Human Gc Gene: Differences and Similarities Between Members of the Albumin Gene Family", pages 751-754, see entire document.	18-20 ----- 1 & 3
X -- Y	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1216, issued 1993, Braun et al, "Sequence and Organization of the Human Vitamin D-Binding Protein Gene", pages 385-394, see entire document.	18 --- 1, 3, 19, & 20
X --- Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, U.S.A., Volume 82, issued December 1985, Yang et al, "Human Group-Specific Component (Gc) is a Member of the Albumin Family", pages 7994-7998, see entire document.	18 --- 1, 3, 19, & 20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 AUGUST 1996

Date of mailing of the international search report

02 OCT 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JEFFREY STUCKER

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08867

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BIOCHEMICA ET BIOPHYSICA ACTA, Volume 871, issued 1986, Schoentgen et al, "Complete Amino Acid Sequence of Human Vitamin D-Binding Protein (Group-Specific Component): Evidence of a Three-fold Internal Homology as in Serum Albumin and alpha-Fetoprotein", pages 189-198, see entire document.	18 --- 1, 3, 19, & 20

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08867

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 3, and 18-20

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08867

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1, 3, and 18-20, drawn to a process for making Gc and to Gc.

Group II, claim(s) 2 and 4, drawn to a process for making GcMAF(c).

Group III, claim(s) 5-7, drawn to a first method of use of GcMAF(c).

Group IV, claim(s) 11 and 12, drawn to GcMAF(c).

Group V, claim(s) 8-10, drawn to a second use of GcMAF(c).

Group VI, claim(s) 13 and 14, drawn to an additional use of GcMAF(c).

Group VII, claim(s) 15-17, drawn to an adjuvant compound.

Group VIII, claim(s) 21, drawn to diagnosing cancer by detecting α -N-acetylgalactosaminidase activity.

Group IX, claim(s) 22, drawn to diagnosing human immunodeficiency virus infection by detecting α -N-acetylgalactosaminidase activity.

Group X, claim(s) 23, drawn to diagnosing cancer by ELISA.

Group XI, claim(s) 24, drawn to diagnosing human immunodeficiency virus infection by ELISA.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The compositions of the starting materials and final products are different from each other. Also, where the methods are the same, the final results of the methods are different. Therefore, the inventions are not so linked by a special technical feature within the meaning of PCT Rule 13.2, so as to form a single general inventive concept.